

## Development of a freeze-stable formulation for vaccines containing aluminum salt adjuvants

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### ABSTRACT

Vaccines containing aluminum salt adjuvants are prone to inactivation following exposure to freeze–thaw stress. Many are also prone to inactivation by heat. Thus, for maximum potency, these vaccines must be maintained at temperatures between 2 °C and 8 °C which requires the use of the cold chain. Nevertheless, the cold chain is not infallible. Vaccines are subject to freezing during both transport and storage, and frozen vaccines are discarded (under the best circumstances) or inadvertently administered despite potentially reduced potency. Here we describe an approach to minimize our reliance on the proper implementation of the cold chain to protect vaccines from freeze–thaw inactivation. By including PEG 300, propylene glycol, or glycerol in a hepatitis B vaccine, particle agglomeration, changes in the fluorescence emission spectrum – indicative of antigen tertiary structural changes – and losses of *in vitro* and *in vivo* indicators of potency were prevented following multiple exposures to –20 °C. The effect of propylene glycol was examined in more detail and revealed that even at concentrations too low to prevent freezing at –10 °C, –20 °C, and –80 °C, damage to the vaccine could be prevented. A pilot study using two commercially available diphtheria, tetanus toxoid, and acellular pertussis (DTaP) vaccines suggested that the same stabilizers might protect these vaccines from freeze–thaw agglomeration as well. It remains to be determined if preventing agglomeration of DTaP vaccines preserves their antigenic activity following freeze–thaw events.

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### 1. Introduction

Maintaining vaccines within proper temperature ranges is important for potency. Unfortunately, the range of temperatures in which a vaccine is stable is often very narrow. Many common vaccines are to be stored between 2 °C and 8 °C, with explicit instructions not to freeze the vaccine [1,2]. Thus, vaccine potency often relies on preventing inadvertent exposure of the vaccine to both elevated and sub-zero temperatures. Deviations outside the proper temperature range in the cold chain have been documented in both developing and developed countries [3–9]. Moreover, efforts to prevent exposing the vaccine to excessive heat, which is often detrimental to the stability of the antigen, can be the culprit for inadvertent freezing [3,5,7,9–11]. The result is often either vaccine wastage or the unintentional administration of suboptimal vaccines.

The need to avoid freezing for maintaining potency of many vaccines that are currently in widespread use (e.g., hepatitis B vaccine, diphtheria toxoid vaccine, tetanus toxoid vaccine, etc.) has been attributed in part to the presence of an aluminum salt adjuvant, which is often required for the vaccine to be effective [12]. This class of adjuvant is the most prevalent adjuvant used in vaccines intended for use in humans. In fact, aluminum salt adjuvants (aluminum hydroxide adjuvant, aluminum phosphate adjuvant, alum) are the only adjuvants that are in USFDA-approved vaccines for humans [13]. Moreover, their long history of use, ready availability, and low cost lends them to be the adjuvants of choice for vaccines for the developing world. When vaccines containing these adjuvants have undergone freezing and thawing, losses in potency have been reported.

Aluminum salt adjuvants agglomerate when subjected to freezing and thawing [12,14,15]. It has been suggested that the reduction in potency following freezing can be attributed to this agglomeration, although a single freeze–thaw event in which the vaccine is held at the temperature for only a short time does not always result in measured losses of antibody titers or *in vitro* potency [14,15]. However, it is possible for a vaccine to be subjected to

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multiple freeze–thaw cycles during its time in the cold chain, and it is also probable that the duration of the exposure at sub-zero temperatures can persist for hundreds of hours [6,8,9]. Our recent systematic study on the effect of freezing temperatures, number of freeze–thaw cycles, duration of exposure to sub-zero temperatures, and agitation on the stability of a hepatitis B vaccine containing an aluminum hydroxide adjuvant suggests that freeze–thaw inactivation of the vaccine following multiple exposures or long durations at sub-zero temperatures is a real danger and that there is a strong correlation between particle agglomeration and reduced potency [14]. Thus, if freeze–thaw induced agglomeration can be avoided, it is anticipated that the potency of the hepatitis B vaccine would be maintained.

We hypothesize that by introducing relatively inexpensive GRAS (generally regarded as safe) polyols to a vaccine formulation, agglomeration can be prevented and potency can be maintained even following repeated excursions to temperatures as low as  $-80^{\circ}\text{C}$ . In this study, we examine three excipients – glycerin, polyethylene glycol (PEG) 300, and propylene glycol – for their protectant properties (e.g., minimizing agglomeration of the vaccine particles, maintaining antigen structure, and maintaining *in vivo* potency even in the absence of a temperature excursion) at a relatively high concentration of 50% (v/v). We then selected one of these excipients, propylene glycol, and prepared hepatitis B vaccine formulations containing various concentrations of this excipient to evaluate the importance of preventing freezing during low temperature ( $-6^{\circ}\text{C}$  to  $-80^{\circ}\text{C}$ ) excursions on maintaining the physical properties of the vaccine and *in vitro* and *in vivo* potency. The selection of propylene glycol as the preferred excipient for further study was based on the results of the 50% study and propylene glycol being present at the highest concentration in an FDA approved intramuscular (IM) injectable drug product (Table 1). Although the goal of the concentration studies was to determine if a concentration of less than 50% propylene glycol could be used, the fact that the FDA database indicates that it is present at extremely high concentrations in approved drugs and our additional literature research revealed 40% propylene glycol

**Table 1**

Maximum amount of PEG 300, propylene glycol, or glycerol as an inactive ingredient in injectable drugs (IM or SC routes)<sup>a</sup>.

Excipient	Route	Maximum amount
Glycerin	IM	15.36%
	SC	32.5%
PEG 300	IM	50%
Propylene glycol	IM	82.4%
	SC	Data not given

<sup>a</sup> FDA/CDER Inactive Ingredient Search for Approved Drug Products Database website. Available at: <http://www.accessdata.fda.gov/scripts/cder/iig/index.cfm>. Accessed September 2, 2008.

concentration in IM drug formulations for pediatric populations (Table 2) provided the highest degree of confidence that this excipient is likely to be well-tolerated at the proposed concentration of this study.

Finally, we conducted a preliminary study with two multivalent (diphtheria, tetanus, and acellular pertussis) vaccines from different manufacturers to determine whether the results that we observed with these excipients and the hepatitis B vaccine might be more universally applied to other vaccines containing aluminum salt adjuvants. This limited study focused on measures of freeze–thaw induced particle agglomeration with and without the potentially protective polyols.

## 2. Materials and methods

### 2.1. Materials

Shanvac-B, a commercially available hepatitis B vaccine, was purchased from Shantha Biotechnics Ltd. (Hyderabad, India). Daptacel (Sanofi Pasteur, Ltd.) and Infanrix (GlaxoSmithKline), two commercially available trivalent DTaP vaccines, were purchased from a doctor's office (Seattle, WA). Buffer salts and thimerosal were purchased from Fisher Chemical Company. ISOTON II and

**Table 2**

Drugs with high PG osmolarity.

Drug	Concentration of PG	Calculated PG osmolarity	Route (injection)	Age of target population
Alkeran <sup>a</sup>	60%	7885 mOsmo/L	IV	Safety not established in pediatric population
Amidate (etomidate) <sup>b</sup>	35%	4600 mOsmo/L	IV	Not recommended for patients below 10 years of age
Diazepam <sup>c</sup>	40%	5257 mOsmo/L	IM or IV	Infants (greater than 1 month) through adult
Lanoxin (digoxin) <sup>d</sup>	40%	5257 mOsmo/L	IV or IM	Infants through adult
Librium (chlordiazepoxide) <sup>e</sup>	20.7%	2720 mOsmo/L	IM	Infants through adult
Lorazepam <sup>f</sup>	18%	2366 mOsmo/L	IM or IV	Safety not established in pediatric population
Nembutal (pentobarbital sodium injection) <sup>g</sup>	40%	5257 mOsmo/L	IV or IM	Pediatric through adult
Nitroglycerin <sup>h</sup>	30%	3943 mOsmo/L	IV	Safety not established in pediatric population
Phenytoin Sodium <sup>i</sup>	40%	5257 mOsmo/L	IM or IV	Pediatric through adult

<sup>a</sup> Alkeran product insert page. GlaxoSmithKline website. Available at: <http://us.gsk.com/products/assets/us.alkeran.injection.pdf>. Accessed May 9, 2007.

<sup>b</sup> Amidate product insert page. Bedford Laboratories website. Available at: [http://www.bedfordlabs.com/products/inserts/etomidate\\_pi.pdf](http://www.bedfordlabs.com/products/inserts/etomidate_pi.pdf). Accessed May 9, 2007.

<sup>c</sup> Diazepam product information page. NIH website. Available at: <http://dailymed.nlm.nih.gov/dailymed/fdaDrugXsl.cfm?id=3743&type=display>. Accessed May 9, 2007.

<sup>d</sup> Lanoxin product insert page. GlaxoSmithKline website. Available at: [http://www.gsk.com/products/prescription\\_medicines/us/lanoxin.htm](http://www.gsk.com/products/prescription_medicines/us/lanoxin.htm). Accessed May 9, 2007.

<sup>e</sup> Librium product information page. Internet Mental Health website. Available at: <http://www.mentalhealth.com/drug/p30-101.html#Head.8>. Accessed May 9, 2007.

<sup>f</sup> Lorazepam product insert page. Baxter Healthcare website. Available at: [http://www.baxter.com/products/anesthesia/anesthetic\\_pharmaceuticals/downloads/lorazepam.pdf](http://www.baxter.com/products/anesthesia/anesthetic_pharmaceuticals/downloads/lorazepam.pdf). Accessed May 9, 2007.

<sup>g</sup> Nembutal product information page. NIH website. Available at: <http://dailymed.nlm.nih.gov/dailymed/fdaDrugXsl.cfm?id=2563&type=display>. Accessed May 9, 2007.

<sup>h</sup> Nitroglycerin product insert page. VaxServe website. Available at: <https://www1.vaxserve.com/views/PDF/Nitroglycerin%20IN4805%20%20Rev10-02.pdf>. Accessed May 9, 2007.

<sup>i</sup> Phenytoin Sodium product information page. NIH website. Available at: <http://dailymed.nlm.nih.gov/dailymed/fdaDrugXsl.cfm?id=615&type=display>. Accessed May 9, 2007.

sample preparation vials for particle sizing were purchased from Beckman Coulter. Biotin-labeled goat anti-mouse IgG H+L and straptavidin-HRP conjugate were purchased from Sigma (St. Louis, MO). Borosilicate vials with butyl rubber stoppers and aluminum seals were used to prepare all vaccine samples.

## 2.2. Vaccine preparation

### 2.2.1. Hepatitis B vaccines

To obtain hepatitis B vaccines with the requisite amount of stabilizer, the vaccine stock was pooled into a glass bottle, and particles were allowed to settle to the bottom of the bottle. Then, 50% of the volume of vaccine supernatant was replaced with appropriate combinations of buffer and excipient. All samples were prepared in triplicate unless otherwise stated. Control vaccine was utilized without further dilution or purification and stored at 4 °C.

### 2.2.2. DTaP vaccines

Samples were prepared in triplicate using 2.0 ml borosilicate glass vials. Briefly, in each 2 ml vial, 1050  $\mu$ l of vaccine (Daptacel or Infanrix) was combined with either 450  $\mu$ l of propylene glycol, PEG 300, or glycerol to result in samples that contained 30% excipient. The 0% additional excipient samples were prepared by combining 1050  $\mu$ l vaccine with 450  $\mu$ l of 0.9% saline. The contents of the vials were gently mixed using the pipette. Vials were capped with 13 mm butyl stoppers. Control samples consisted of vaccine as supplied and simply transferred to 2.0 ml borosilicate glass vials, capped, and stored at 4 °C until assayed.

## 2.3. Freeze–thaw treatments—hepatitis B vaccine

### 2.3.1. 50% excipient

To screen the effectiveness of 50% propylene glycol, PEG 300, or glycerin on preventing damage to the hepatitis B vaccine, the vaccine formulations were exposed to three freeze–thaw cycles of  $-20^{\circ}\text{C}/\text{RT}$ . For each freezing cycle, the vials were placed in a  $-20^{\circ}\text{C}$  freezer (Amana) overnight for at least 12 h. Room temperature thawing was achieved by placing the vials on the lab bench for approximately 2 h.

### 2.3.2. 20% propylene glycol

Samples treated to a temperature of  $-6^{\circ}\text{C}$  or  $-10^{\circ}\text{C}$  were placed in an environmental chamber (LH1.5, Associated Environmental Systems, Ayer, MA) that had been set to the desired temperature and allowed to equilibrate at that temperature for 1 h prior to loading the samples. The temperature fluctuation range of the chamber was  $\pm 0.5^{\circ}\text{C}$  and the actual temperature was confirmed by placing a thermometer inside the chamber. Samples treated to  $4^{\circ}\text{C}$  or  $-20^{\circ}\text{C}$  were placed in a freezer-less refrigerator or a standalone freezer set to  $-20^{\circ}\text{C}$ , and the temperatures of both were verified daily using thermometers placed inside the chambers. All samples for a given temperature treatment were placed in the appropriate chamber at the same time. After the first hour, the samples exposed to the sub-zero temperatures were inspected for signs of freezing. The vials were then gently tapped once on the side with a metal rod and inspected for freezing again to account for the possibility of supercooling. The vials were then returned to the appropriate chamber. After 6 h (total time), six vials of vaccine without additional propylene glycol were removed from each of the sub-zero storage chambers, placed on the lab bench (at approximately  $20^{\circ}\text{C}$ ) for 2 h, then placed into the  $4^{\circ}\text{C}$  refrigerator until analyzed. The remaining vials containing vaccine or vaccine and 20% propylene glycol were removed from the sub-zero storage 24 h after the initial placement in the chamber and placed

on the lab bench for 2 h. This freeze–thaw cycle (24 h freezing/2 h thawing, with tapping after 1 h) was repeated two additional times for a total of three freeze–thaw cycles. After the final freeze–thaw cycle, the vials were placed at  $4^{\circ}\text{C}$  until analyzed. Vaccine without additional stabilizer and subjected only to storage at  $4^{\circ}\text{C}$  served as the control. There were six vials per treatment and formulation.

### 2.3.3. Propylene glycol concentration study at $-10^{\circ}\text{C}$

For these studies, the vaccine formulations were subjected to three freeze–thaw treatments of  $-10^{\circ}\text{C}$  and  $4^{\circ}\text{C}$ . The studies were conducted as previously described except each part of the cycle, freezing or thawing, was overnight for a minimum of 18 h. The samples were held at  $4^{\circ}\text{C}$  following the thermal treatment until assayed.

### 2.3.4. Propylene glycol concentration study at $-20^{\circ}\text{C}$ and $-80^{\circ}\text{C}$

Hepatitis B vaccine formulations with 0%, 10%, or 30% propylene glycol were subjected to either three or six freeze–thaw cycles at either  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$ . In both cases, exposure to the freezing temperature was accomplished by placing the vials in  $-20^{\circ}\text{C}$  and  $-80^{\circ}\text{C}$  laboratory freezers.

## 2.4. Freeze–thaw treatments—DTaP vaccines

Samples were frozen by placing the vials at  $-20^{\circ}\text{C}$  for 24 h. After the first hour at  $-20^{\circ}\text{C}$ , each vial was briefly removed from the freezer for the purposes of tapping the side of the vial once with a rod to facilitate the freezing of any potentially supercooled samples. The vials were then returned to the  $-20^{\circ}\text{C}$  freezer. At the end of the 24-h incubation at  $-20^{\circ}\text{C}$ , the samples were removed from the freezer and allowed to thaw at room temperature for 2 h. Samples were then placed at  $4^{\circ}\text{C}$  until assayed.

## 2.5. Particle sizing

Particle sizing was conducted using a Coulter Counter (model Z1, Beckman Coulter, Fullerton, CA). ISOTON II served as the diluent. Measurements of the diluent without sample served as the background. The samples were assayed for particles using the following size intervals: 1.5–3  $\mu\text{m}$ , 3–6  $\mu\text{m}$ , 6–9  $\mu\text{m}$ , 9–15  $\mu\text{m}$ , 15–20  $\mu\text{m}$ , 20–25  $\mu\text{m}$ , and 25–30  $\mu\text{m}$ . For each size interval, six readings of each sample were averaged. This averaged value was recorded for use as one of the triplicate sample values. The average of three separate samples per group (formulation and treatment) were then tabulated in terms of number of particles which was converted to percentage of the total population.

## 2.6. Freezing point determination

The freezing point of each sample was determined using a differential scanning calorimeter (Diamond DSC, PerkinElmer Diamond, Waltham, MA). Indium was used as the calibration standard. Each sample was prepared by placing 20  $\mu$ l of sample in an aluminum DSC pan. The capped pan was then placed in the chamber and subjected to a thermal cycle. The thermal cycle consisted of the following:

- (1) heat to  $50^{\circ}\text{C}$  at  $10^{\circ}\text{C}/\text{min}$ ,
  - (2) hold at  $50^{\circ}\text{C}$  for 1 min,
  - (3) cool to  $-40^{\circ}\text{C}$  at  $10^{\circ}\text{C}/\text{min}$ ,
  - (4) heat to  $50^{\circ}\text{C}$  at  $10^{\circ}\text{C}/\text{min}$ ,
  - (5) hold for 1 min at  $50^{\circ}\text{C}$ ,
  - (6) cool to  $-40^{\circ}\text{C}$  at  $10^{\circ}\text{C}/\text{min}$ .
- The melting/freezing point was determined during the heating phase from  $-40^{\circ}\text{C}$  to  $50^{\circ}\text{C}$ .

### 2.7. Fluorescence spectroscopy to monitor antigen conformational changes in the hepatitis B vaccine

Samples (1.8 ml each) were transferred via pipette into triangular fluorescence cuvettes and allowed to settle overnight (minimum 16 h) at 4 °C. The next day, fluorescence spectra were obtained. The cuvettes were positioned in the fluorometer such that the excitation beam hit the antigen/adjuvant layer that had formed in the bottom portion of the cuvette. Samples were excited with a wavelength of 280 nm and emission spectra were obtained to monitor the fluorescence of the antigen's tyrosine and tryptophan residues. All spectra were collected at 25 °C using a QM-4 fluorometer (Photon Technology International, Inc., Birmingham, NJ) with a Peltier temperature-controlled cuvette holder (Quantum Northwest, Shoreline, WA). To correct for artifacts arising from the cuvette, fluorescence spectra of vaccine-free samples with the appropriate excipient in the corresponding sample's cuvette were also collected.

The emission peak positions of the fluorescence spectra were determined using derivatives. Briefly, following subtraction of the blank spectrum, a 7-pt Savitsky Golay smoothing algorithm was applied and the first derivative of the spectrum was obtained (Origin 7, OriginLab Corp., Northampton, MA). To enhance visualization of the shifts, spectra were normalized to an arbitrary height of 1 using the Origin software and overlaid. The statistical significance of the differences in the observed peak positions were determined by performing a one-way ANOVA analysis with Dunnett posttest on the data (Prism, GraphPad Software Inc., La Jolla, CA).

### 2.8. *In vitro* potency assay

The *in vitro* potency assay (AUZYME) of the hepatitis B vaccine was performed according to the manufacturer's (Abbott Laboratories, Abbott Park, IL) instruction as previously described [14]. The vaccine was centrifuged at 1500 rpm for 10 min to pellet the adjuvant. The supernatant was then assayed to determine the amount of HBsAg that dissociated from the adjuvant. Samples containing known concentrations of HBsAg were used to produce a standard curve.

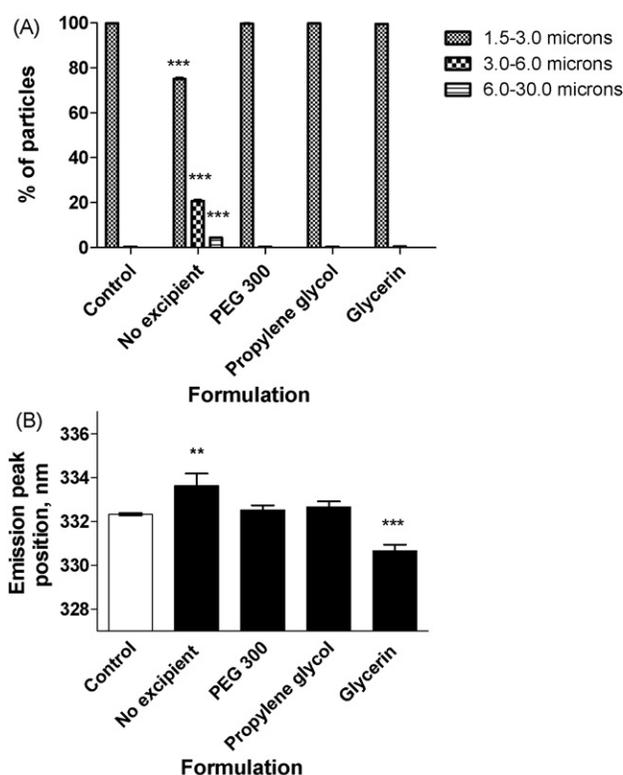
### 2.9. *In vivo* potency assay

For each of the immunogenicity results described, eight 5- to 7-week-old BALB/c mice (Spring Valley Laboratory, Woodbine, MD) per formulation/treatment condition were used. Mice were dosed via intra-peritoneal injections as previously described. Blood was collected (150–250 µl from each mouse) on day 43 for determination of antibody concentration to HBsAg using an ELISA as previously described [14].

## 3. Results

### 3.1. Effects of 50% PEG 300, propylene glycol, or glycerol on vaccine freeze–thaw stability

In the absence of any additional excipients, Shanvac-B vaccine visually froze when stored at –20 °C. The addition of 50% PEG 300, propylene glycol, or glycerol prevented visually observable freezing. Agglomeration and sedimentation rates, two standard methods of assessing whether a vaccine containing an aluminum salt adjuvant has undergone freeze–thaw damage, also indicated that 50% excipient prevented the vaccine from freezing. Samples that were frozen and thawed without the added excipients were heavily agglomerated and had measurable sedimentation after 60 min. PEG

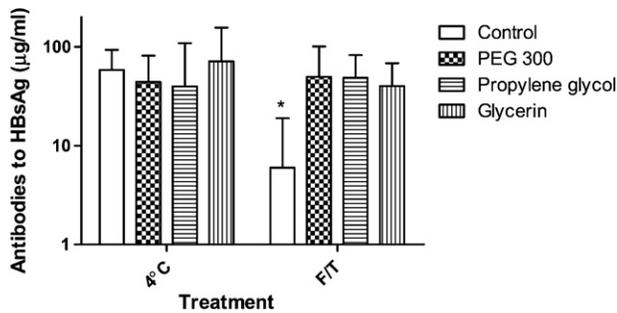


**Fig. 1.** Effect of 50% (v/v) PEG 300, propylene glycol, or glycerin on hepatitis B vaccine (A) particle size distribution as measured using a Coulter Counter and (B) fluorescence emission peak position, usually indicative of protein conformational changes, following three –20 °C/RT freeze–thaw cycles. (Results of one-way ANOVA analyses with Dunnett posttest to compare the mean values to the untreated control: \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .)

300, propylene glycol, and glycerin prevented agglomeration and measurable sedimentation within that same time frame.

It is possible that the reduced sedimentation was primarily due to the increased viscosity of the samples containing the PEG 300, propylene glycol, or glycerin. Thus, the particle size distributions were also determined using a Beckman Coulter Counter. As shown in Fig. 1A, initially over 99% of the vaccine particles were between 1.5 µm and 3 µm. Following the freeze–thaw treatment, the percentage of particles in this size range reduced to approximately 75% in the absence of the excipients. Over 20% of the particles in the “no excipient” sample were between 3 µm and 6 µm, compared to less than 1% in the control samples. The vaccine that was frozen and thawed without additional excipients had measurable particles in all ranges through 25–30 µm. PEG 300, propylene glycol, and glycerol (50%) had particle size distributions that were nearly indistinguishable from the control.

We have previously reported that freezing and thawing also cause an apparent conformational change in the adsorbed hepatitis B surface antigen (HBsAg), indicated by a small but significant red shift in the intrinsic fluorescence emission spectrum [14]. Under the conditions for the current study, this shift was again observed (Fig. 1B). Similarly to the agglomeration results, the emission peak positions of the hepatitis B vaccine formulations containing PEG 300 (50%) or propylene glycol (50%) were indistinguishable from the control (Fig. 1B). In contrast, the 50% glycerin formulation had a statistically significant blue shift in the spectrum. In the absence of the freeze–thaw treatment, preliminary studies did not reveal solvent-induced peak shifts when propylene glycol, PEG 300, or glycerin was included in the formulation (data not shown).



**Fig. 2.** HBsAg antibodies produced by balb/c mice following a prime/boost inoculation of hepatitis B vaccine as manufactured (control) or containing 50% (v/v) PEG 300, propylene glycol, or glycerin and either stored at 4 °C or subjected to three –20 °C/RT freeze–thaw cycles. (Statistical analysis compares the average values to the 4 °C control—no excipient; \* $p < 0.05$ .)

### 3.2. Effects of 50% stabilizer on antibody production

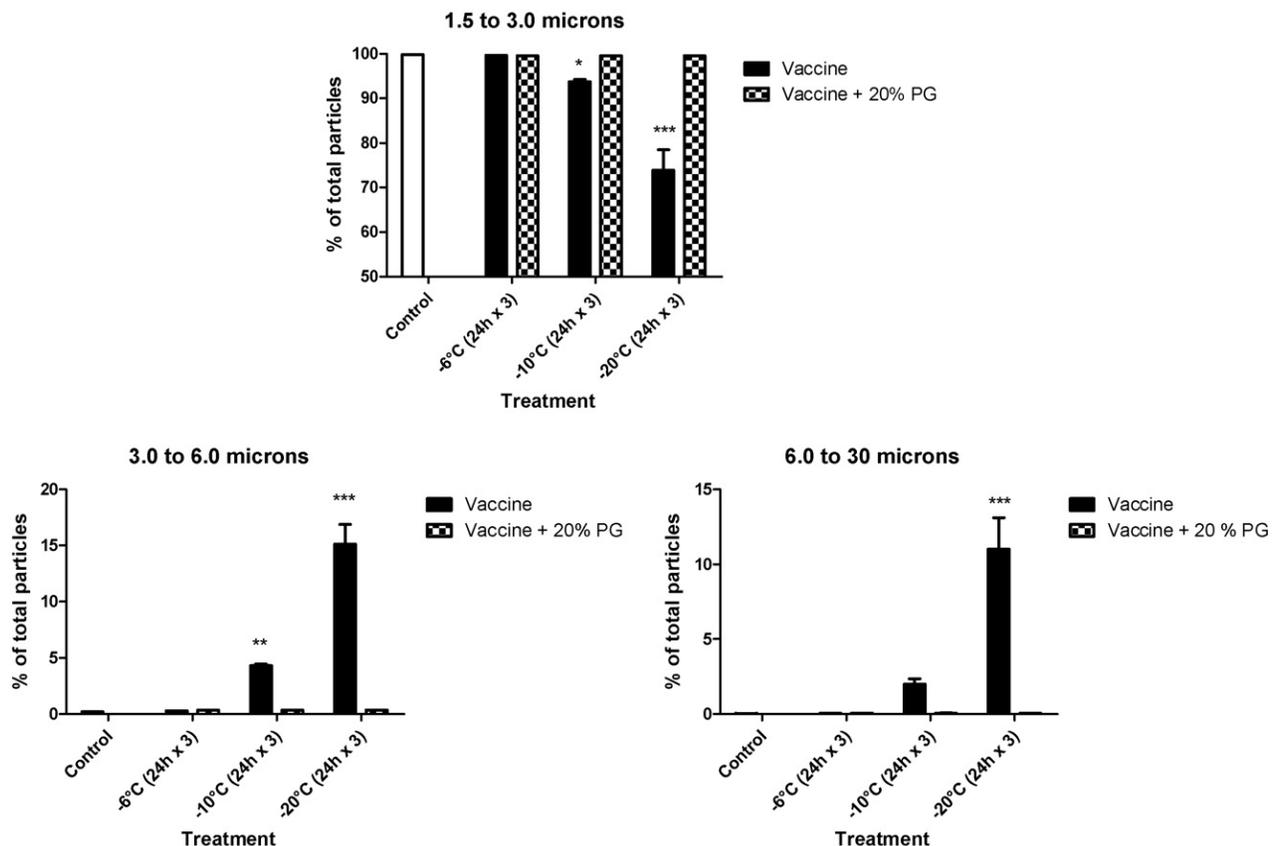
Ultimately, preserving immunogenicity is the primary concern. Antibody titers in mice were used as indicators to determine whether excipients added at concentrations to inhibit freezing of the vaccine exposed to –20 °C would also inhibit loss of vaccine immunogenicity. Simply adding the excipients to the vaccine that was stored at 4 °C had no effect on total antibody production (Fig. 2). Following three exposures to –20 °C, the vaccine lacking the additional excipients resulted in significantly reduced antibody titers, as expected from our previous studies [14]. Levels of antibody to

HBsAg in mice that were administered any of the vaccines to which the excipients were added prior to the freeze–thaw treatment were indistinguishable from the 4 °C control (Fig. 2).

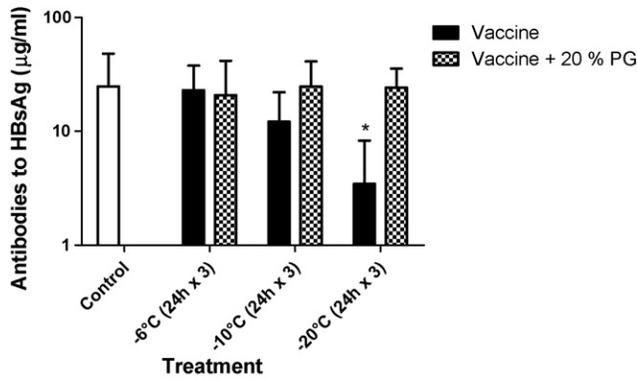
### 3.3. Effectiveness of 20% propylene glycol

#### 3.3.1. Reduction of the equilibrium freezing point

The equilibrium freezing point of the original hepatitis B vaccine tested was approximately –2 °C. If it is necessary to prevent freezing to inhibit freeze–thaw damage, the vaccine must contain sufficient stabilizer to depress the equilibrium freezing point below that of the lowest temperature to which the vaccine might likely be exposed. Recent cold chain studies in several countries have reported vaccines being inadvertently exposed to temperatures as low as –13 °C during transport [8,9]. Although a recent study of the vaccine cold chain in Bolivia did not reach temperatures below –10 °C, the minimum temperature of exposure during all parts of the cold chain monitored were all at or below the equilibrium freezing point of our hepatitis B vaccine and ranged from –2.2 °C to –7.2 °C [6]. Using differential scanning calorimetry, we determined that the 50% propylene glycol formulation had an equilibrium freezing point of approximately –32 °C. Although the equilibrium freezing measurement suggests that 50% propylene glycol should provide protection against freezing at the most commonly encountered sub-zero temperatures, formulation and osmolarity considerations would recommend that the concentration of freeze protectant be lowered. By lowering the concentration to 20% propylene glycol, the equilibrium freezing point of the formulation would be approximately –15 °C. Thus, by using 20%



**Fig. 3.** Agglomeration of hepatitis B vaccine particles for vaccine formulated with and without 20% propylene glycol following three freeze–thaw exposures with low temperatures of –6 °C, –10 °C, or –20 °C. (Results of a two-way ANOVA analysis with Bonferroni posttest to compare values to the untreated control: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .)



**Fig. 4.** HBsAg antibody production in mice administered the vaccine formulations from Fig. 3. (Results of a two-way ANOVA analysis with Bonferroni posttest to compare values to the untreated control: \* $p < 0.05$ .)

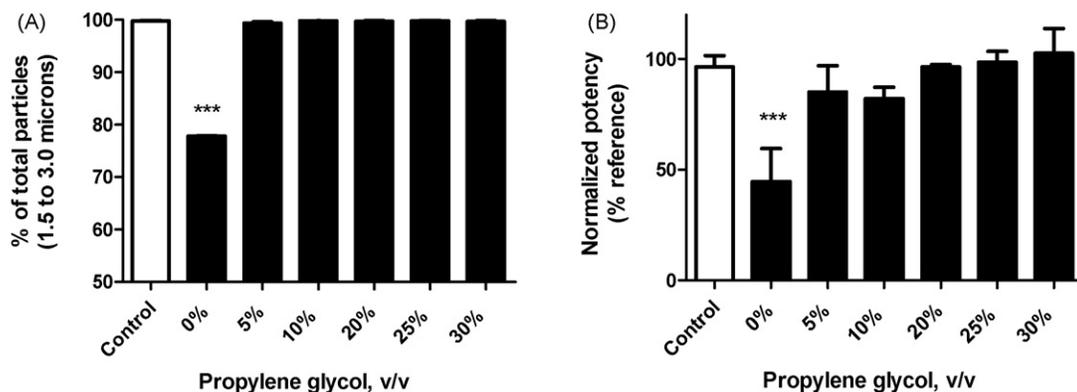
propylene glycol, the freezing point of the vaccine is now well below temperatures most commonly encountered.

### 3.3.2. Effectiveness at temperatures above and below the equilibrium freezing point

As observed in our previous study at  $-10^{\circ}\text{C}$ , a single exposure to the sub-zero temperatures of  $-6^{\circ}\text{C}$ ,  $-10^{\circ}\text{C}$ , or  $-20^{\circ}\text{C}$  for 6 h did not result in measurable particle agglomeration (data not shown). Following three cycles of exposure to  $-6^{\circ}\text{C}$  and room temperature of approximately  $20^{\circ}\text{C}$ , the vaccine independent of propylene glycol content did not freeze, agglomerate (Fig. 3), or suffer any loss in the ability to induce antibody production in mice (Fig. 4). In contrast, three freeze–thaw cycles at temperatures down to  $-10^{\circ}\text{C}$  or  $-20^{\circ}\text{C}$  caused significant agglomeration of the vaccine lacking the propylene glycol, but the 20% propylene glycol formulation did not agglomerate (Fig. 3). At temperatures of  $-10^{\circ}\text{C}$  and  $-20^{\circ}\text{C}$ , the vaccine lacking the propylene glycol froze. Propylene glycol (20%) was sufficient to prevent the vaccine from freezing at  $-10^{\circ}\text{C}$  but not at  $-20^{\circ}\text{C}$ . The only formulation that resulted in a significant reduction of antibody production was the vaccine without the propylene glycol that was subjected to three freeze–thaw cycles down to  $-20^{\circ}\text{C}$  (Fig. 4).

### 3.4. Propylene glycol concentration effect on the stability of vaccine subjected to three freeze–thaw cycles at $-10^{\circ}\text{C}$ and $4^{\circ}\text{C}$

A concentration dependence study was conducted to address the question of the minimum concentration of propylene glycol



**Fig. 5.** Effect of propylene glycol concentration on the (A) agglomeration of the hepatitis B vaccine particles as measured by a Coulter Counter and (B) *in vitro* potency of the HBsAg vaccine as measured by the AUSZYME assay following freeze–thaw treatment. (Results of one-way ANOVA analyses with Dunnett posttest to compare the mean values to the untreated control: \*\*\* $p < 0.001$ .)

**Table 3**

Equilibrium freezing point of the hepatitis B vaccine formulated with propylene glycol or PEG 300: concentration dependence.

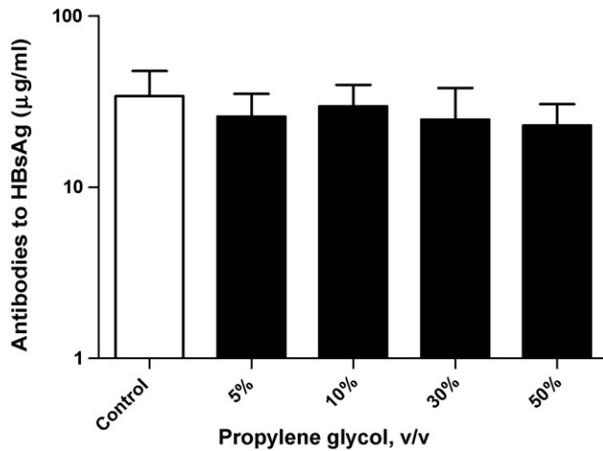
Formulation = hepatitis B vaccine +	Freezing point ( $^{\circ}\text{C}$ ) <sup>a</sup>
Nothing (control)	-1.9
5% propylene glycol	-2.4
10% propylene glycol	-8.9
15% propylene glycol	-11.7
20% propylene glycol	-15.2
15% PEG 300	-7.5
20% PEG 300	-9.4
25% PEG 300	-11.7
30% PEG 300	-14.1

<sup>a</sup>  $n = 1$

that would be required to prevent damage following repeated exposures to a temperature at which the original vaccine freezes. Of the concentrations examined, propylene glycol concentrations  $\leq 10\%$  were insufficient to prevent freezing at  $-10^{\circ}\text{C}$ . This observation is supported by a preliminary study to examine the equilibrium freezing point as a function of the concentration of propylene glycol or PEG 300 – the only potential stabilizers that at 50% inhibited agglomeration and shifts in the intrinsic fluorescence emission spectrum of the vaccine following the freeze–thaw treatment – in the vaccine formulations (Table 3). In the present study, the vaccine remained liquid only when the concentration was 20% or higher. Nevertheless, as little as 5% propylene glycol prevented detectable agglomeration of the vaccine particles (Fig. 5A). This low concentration was also sufficient to prevent loss of *in vitro* potency (Fig. 5B). In a separate study, the immunogenicity of hepatitis B vaccine in mice was assessed using low concentrations of propylene glycol. Antibody production using the vaccine exposed to three freeze–thaw cycles at  $-10^{\circ}\text{C}$  and  $4^{\circ}\text{C}$  and formulated with as little as 5% propylene glycol is similar to the control vaccine stored at  $4^{\circ}\text{C}$  (Fig. 6).

### 3.5. Effect of low concentrations of propylene glycol on the stability of vaccines exposed to $-20^{\circ}\text{C}$ or $-80^{\circ}\text{C}$

Although temperatures of approximately  $-10^{\circ}\text{C}$  and higher would be expected to be most commonly encountered [6,8,9], accidental placement of the vaccine in commercial freezers could result in the vaccine being exposed to temperatures as low as  $-80^{\circ}\text{C}$ . Three or six freeze–thaw cycles with temperature excursions to either  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$  resulted in reduction in both the percentage of particles in the ideal size range (Fig. 7A) and potency (Fig. 7B). Increasing the number of freeze–thaw cycles from three to six did not cause appreciable additional damage. If propylene glycol (10%



**Fig. 6.** Effect of 5–50% propylene glycol on the production of HBsAg antibodies in mice immunized with HBsAg vaccine that had been subjected to three  $-10^{\circ}\text{C}/4^{\circ}\text{C}$  freeze–thaw cycles. (One-way ANOVA analyses with Dunnet posttest to compare the mean values to the untreated control revealed no statistically significant differences.)

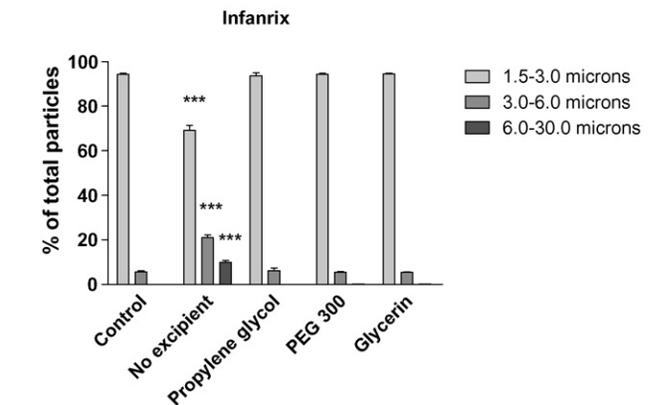
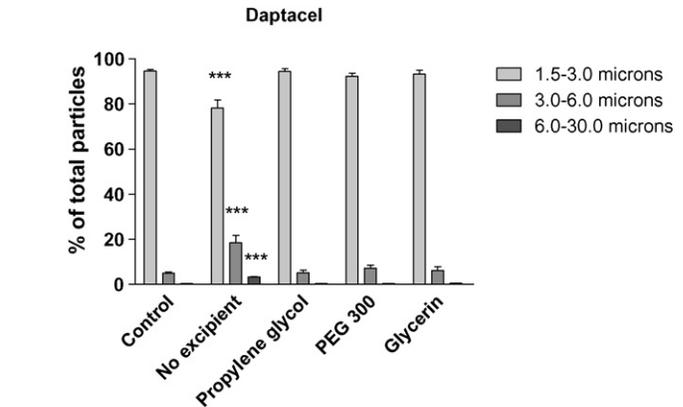
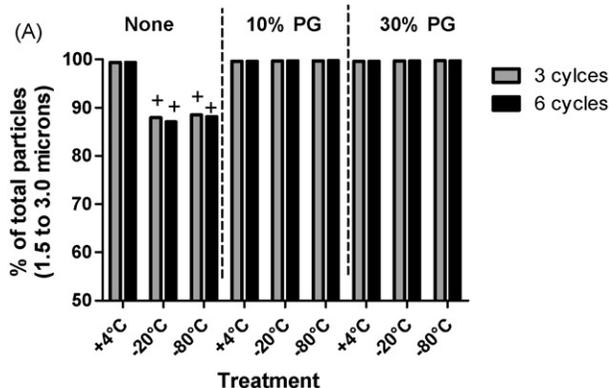
or 30%) was included in the formulation, the agglomeration and potency loss were prevented.

### 3.6. Effect of stabilizers on the freeze–thaw agglomeration of diphtheria, tetanus, and acellular pertussis (DTaP) vaccines

DTaP vaccines containing either aluminum phosphate adjuvant or aluminum hydroxide adjuvant were reformulated to include 30% PEG 300, propylene glycol, or glycerin. As with the HBsAg vaccine, the original vaccine formulations agglomerated following the freeze–thaw treatment. Aggregation of the vaccine particles was inhibited by the excipients (Fig. 8).

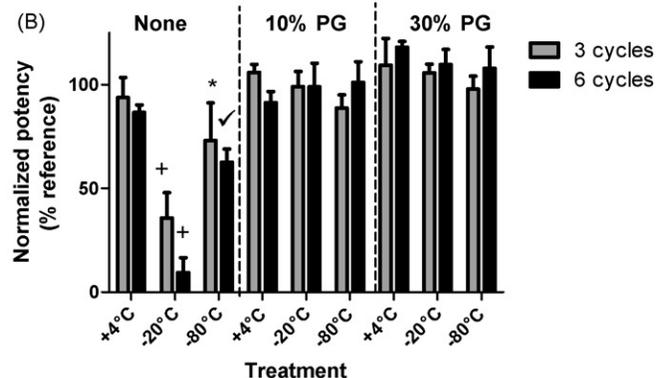
## 4. Discussion

Despite the availability of equipment and practices that could be implemented today to minimize the risks of inadvertent freezing of vaccines containing aluminum salt adjuvants, the risks are still very real in both developed and developing countries [3–9]. This has the potential for grave consequences because freeze–thaw damaged vaccines are likely to be less effective than vaccines that have been properly stored until the point of administration. More robust vaccine formulations are likely to be the best defense against the risks of inadvertent vaccine freezing since most other



**Fig. 8.** Effect of 0% or 30% propylene glycol, PEG 300, or glycerin on the agglomeration of two commercial DTaP vaccines subjected to a single freeze–thaw treatment at  $-20^{\circ}\text{C}$ . (A) Daptacel and (B) Infanrix. (Results of one-way ANOVA analyses with Dunnet posttest to compare the mean values to the untreated control:  $***p < 0.001$ .)

strategies require human compliance for success. It is important, however, that any method to achieve new vaccine formulations takes an approach that is sensitive to (1) the cost of implementation and (2) the perceived safety of the additional excipients. Here we have described the results of a study that identifies excipients capable of preserving measured physical and biophysical characteristics of a vaccine containing an aluminum salt while meeting the above criteria to minimize the resistance of reformulation.



**Fig. 7.** Effect of 0%, 10%, or 30% propylene glycol on the vaccine: (A) percentage of particle remaining in the 1.5–3.0  $\mu\text{m}$  size range and (B) *in vitro* potency following three or six freeze–thaw cycles with freezing temperatures of either  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$  (Results of a two-way ANOVA analysis with Bonferroni posttest to compare values to the  $4^{\circ}\text{C}$  control:  $*p < 0.05$ ,  $\sqrt{p} < 0.01$ ,  $*p < 0.001$ .)

In this study, we have demonstrated that three generally regarded as safe excipients – PEG 300, propylene glycol, and glycerin – at a concentration of 50% prevented agglomeration of a hepatitis B vaccine formulated with an aluminum hydroxide adjuvant and maintained the ability of the vaccine to stimulate antibody production in mice. In this case, the preserved activity can be attributed to the high concentration of excipients inhibiting the freezing of the vaccine at  $-20^{\circ}\text{C}$  by depressing the thermodynamic freezing point but seeming to have no effect, adverse or beneficial, on the activity of the vaccine in the absence of freezing. PEG 300 and propylene glycol, unlike glycerin, also resulted in vaccine formulations with fluorescence spectra, often used to detect changes in a protein's tertiary structure, similar to the  $4^{\circ}\text{C}$  control.

The investigation of the effect of propylene glycol at a range of concentrations indicated that at least in the case of this excipient, concentrations too low to prevent the vaccine from freezing could still inhibit particle agglomeration, alteration in protein structure, and loss of *in vitro* and *in vivo* activity following exposure to freezing temperatures. Propylene glycol at 5% was sufficient to protect the vaccine from damage following multiple freeze–thaw cycles with a low temperature of  $-10^{\circ}\text{C}$ , and as little as 10% propylene glycol prevented any measurable damage during multiple freeze–thaw cycles when the low temperature was  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$ . This was an important finding since it is desirable to minimize the amount of stabilizer added to the vaccine formulation from the standpoint of minimizing the increase in the osmolality of the vaccine and cost of materials (which in turn affects the cost of the finished product).

We were also able to generate preliminary evidence that suggests that these excipients may have general utility as freeze–thaw stabilizers for other vaccines containing an aluminum salt adjuvant. Commercially available DTaP vaccines from two different manufacturers demonstrated conservation of particle size distribution following the freeze–thaw treatment if the vaccines were formulated with 30% (v/v) of any of the three excipients used in this study. Future studies are needed to confirm the preservation of antigen structure and immunogenicity.

One important question that is not addressed in the current study is what is the effect of these excipients long-term (i.e., on the order of months or years) on the stability of these vaccines, stored either at  $4^{\circ}\text{C}$  or at freezing temperatures. In addition, although some temperature monitoring studies suggest that accidental exposure to freezing temperatures may be significantly more frequent and/or longer than inadvertent exposure to elevated temperatures [6,8,9], the potential consequences of the presence of these excipients on vaccines containing an aluminum salt adjuvant following a temperature excursion above room temperature also

demand exploration. Nevertheless, the results of this study examining the short-term consequences of including propylene glycol at various concentrations or PEG 300 or glycerin at concentrations of 50% (v/v) (hepatitis B vaccine) or 30% (DTaP vaccines) for vaccines exposed to freezing temperatures suggest a potential solution to a problem with vaccines that contain aluminum salt adjuvants that has plagued both vaccine manufacturers and health care providers for many years.

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